GM-CSF Inhibits Breast Cancer Growth and Metastases in Mice and Induces Hypoxia in the Tumor Proper

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BACKGROUND

Malignant cancers require the cooperation of otherwise normal cells that surround and invade tumors in order to grow and spread. Tumors must not only evade the host defense, but also maintain a sufficient blood supply to support rapid growth and navigate a complex series of cell-cell interactions required to undergo successful metastasis (Hanahan, D. and Weinberg, R. A., 2000). This requires that tumors find ways to exploit normal host cells, causing them to adopt behaviors which permit and even promote malignant transformation.

Different tumor types face different obstacles they must overcome to become malignant. These challenges arise from the nature of the cells themselves and the tissues in which they reside. Diversity exists both in the native characteristics of the tumor cells themselves and in the mechanisms they employ to overcome these barriers to malignancy. Some tumors routinely downregulate immune surveillance molecules so that they may avoid attacks by cytotoxic T cells and natural killer (NK) cells (Watson, N. F. et al., 2006; Tsuruma, T. et al., 1999). Others secrete high levels of factors which stimulate blood vessel growth (Demirkesen, C. et al., 2006). Others downregulate molecules which maintain interactions with other cells (Oka, H. et al.,

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1993). Many of these behaviors evoked by "tumor education" represent an inappropriate triggering of native developmental programs within the otherwise normal cells (Lotem, J. and Sachs, L., 2002).

One type of immune cell, the macrophage, plays an important role both in the development of normal breast tissue and in the development and spread of breast tumors. Macrophage activity, stimulated by the aptly named macrophage colony stimulating factor (M-CSF), is essential for normal breast development, especially during pregnancy and lactation (Pollard, J. W., 1997). Macrophages are the most abundant immune cell in breast tumors, constituting up to 35% of tumor-infiltrating inflammatory cells (Tang, R. et al., 1992). These tumor associated macrophages (TAMs) produce factors that facilitate tumor invasion and angiogenesis, including matrix metalloproteinases (MMPs, (Hagemann, T. et al., 2004)) and VEGF (Eubank, T. D. et al., 2003).

The millieu of cytokines secreted by tumor cells, stromal cells, and infiltrating immune cells dictates the behavior of macrophages within the tumor and determines the factors they will produce. Many tumors secrete M-CSF (Sapi, E., 2004), and this appears to be the predominant macrophage-trophic factor within most breast tumors. Landmark studies showed that mice deficient in M-CSF were protected against breast tumor metastasis, and that metastatic activity could be restored by expressing M-CSF solely in the breast tissue (Lin, E. Y. et al., 2001). We showed that M-CSF stimulates monocyte/macrophages to produce VEGF, which is biologically active and stimulates angiogenesis (Eubank, T. D. et al., 2003). VEGF (vascular endothelial growth factor) is a very important factor for tumor growth—it is one of the principal factors responsible for stimulating blood vessel growth (angiogenesis). Tumors which cannot generate VEGF or another stimulus for angiogenesis cannot grow beyond $2mm^3$ (Folkman, J., 1990).

This process by which tumor cells commandeer “normal” macrophages to perform activities that promote tumor malignancy have been termed “tumor education” (Pollard, J. W., 2004).

In sharp contrast to M-CSF, GM-CSF-stimulated monocytes exhibit several anti-tumor behaviors. GM-CSF enhances the ability of macrophages to present antigen and initiate an immune response (Armstrong, C. A. et al., 1996). It also stimulates monocytes to secrete large amounts of a soluble VEGF receptor, which binds to and inactivates VEGF present in the millieu (Eubank, T. D. et al., 2004). These observations have led us to speculate that we might manipulate macrophage behavior *in vivo* by altering the milieu of factors that surround them. We propose that the effects of locally-administered GM-CSF may alter the behavior of TAMs, reversing the effects of tumor education and inducing an anti-tumor phenotype (**Figure 1**). Successful application of this approach would prove the biologic plausibility of microenvironmental manipulation as a therapeutic modality for solid tumors.

METHODS

*Mice***.** All procedures involving mice were conducted in accordance with OSU ILACUC protocol regarding the use and care of experimental animals. The PyMT transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mammary tumors from the PyMT transgenics were removed and orthotopically injected into normal FVB/N female mice for these studies.

*Tumor injections***.** Primary mammary tumors from female PyMT mice were removed, homogenized using trypsin, filtered and cultured in DMEM containing 10% FBS, 10 μg/ml insulin, and 5 ng/ml rhEGF in flasks until 80% confluent. The cells were trypsinized, washed, and resuspended in DMEM media at 500,000 cells per 100 μl. The cell suspension was drawn

into insulin syringes and orthotopically injected into the number four mammary fat pads of normal female FVB/N mice.

*Treatment study***.** After the tumors became palpable (about 4 weeks), either PBS or 1, 10 100, or 1000 ng rmGM-CSF in 50 μls was administered using an insulin syringe directly into the tumor. A total of seven treatments were administered (three times per week). The largest tumor dimension and mouse weight was measured weekly. For studies analyzing the effect of neutralizing sVEGFR-1 in combination with GM-CSF treatment, tumors were orthotopically injected in the same manner. After they became palpable, either PBS, 100 ng rmGM-CSF, 100 ng rmGM-CSF + 4 μg anti-VEGF receptor-1 neutralizing antibody, 100 ng rmGM-CSF + 4 μg isotype IgG control (goat), or 4 μg anti-VEGF receptor-1 neutralizing antibody in 50 μl was injected directly into the tumors using insulin syringes.

*EPR oximetry***.** Lithium octa-n-butoxy 2,3-naphthalocyanine (LiNc-BuO) microcrystals were a gift from Dr. Periannan Kuppusamy, The Ohio State University. The PyMT tumor cells were prepared as the previous treatment study. 10 mg microcrystals were resuspended in 500 μl DMEM and vortexed copiously. 25 μl of this suspension was added to $5x10^5$ PyMT cells for each 100 μl injection. Oxygen measurements were performed immediately, and then weekly following treatment with rmGM-CSF using *in vivo* EPR oximetry. Measurements of tumor oxygenation was performed non-invasively using an L-band *in vivo* EPR spectrometer (L-band, Magnettech, Germany) equipped with automatic coupling and tuning controls. Mice, under anesthesia (2% isofluorane), were placed in a right and left-lateral positions with their tumor close to the loop of the surface coil resonator. EPR spectra were acquired as single 30-sec scans. The instrument settings were: incident microwave power, 4 mW; modulation amplitude, 180

mG, modulation frequency 100 kHz; receiver time constant, 0.2 s. The peak-to-peak width of the EPR spectrum was used to calculate pO2 using a standard calibration curve.

*Evaluation of angiogenesis***.** Texas red–conjugated dextran (mol wt 70,000; Molecular Probes, Eugene, OR) was prepared to 6.2 mg/mL in PBS and was injected i.v. via the tail vein at 21 μg/g of mouse body weight. The mice were humanely sacrificed for tissue preparation 5 minutes after the injection. Blood was drawn for circulating blood cell analysis by heart stick. Each tumor was removed and cut in half - one half was placed into a cryovial and flash frozen in liquid nitrogen for cytokine expression within the extracellular fluid, the other half was formalin fixed for immunohistochemical staining. Tumor sections were analyzed for fluorescence using confocal microscopy and functional blood vessels were identified and quantified using Adobe Photoshop CS2.

*Immunohistochemistry***.** For identification of cell types within the tumors, the formalin-fixed, paraffin-embedded mammary tumors were stained to identify CD34+ (endothelial cells), F4/80+ (macrophages), and LY6G+ (neutrophils) cells, as well as hematoxylin & eosin (H&E) for tissue morphology. Total cell influx was analyzed by digital images captured from the entire tumors and quantified using Adobe Photoshop CS2 software. All immunohistochemistry was performed at the Histology Core at The Ohio State University.

*Evaluation of necrosis***.** Upon euthanasia, the lungs from mice treated with GM-CSF or PBS were removed, insufflated, fixed in formalin and paraffin embedded, sectioned and stained with hematoxylin & eosin. Necrotic tissue within the tumors was evaluated by capturing digital images using a 1x objective and exposure to green fluorescent light as described (Achilles, E. G. et al., 2001). Necrosis was quantified using Adobe Photoshop CS2 software.

*Tumor metastases***.** Upon euthanasia, the lungs from mice implanted with tumor cells and treated with GM-CSF or PBS were removed, insufflated, fixed in formalin, and stained with eosin. Tumor cell metastases were evaluated by subjecting the lungs to Bright Field light under a stereomicroscope. Each tumor incident was counted in a blinded manner.

RESULTS

*Local treatment with GM-CSF slows tumor growth in a mouse model of breast cancer***.** Based on the results of our in vitro work (Eubank, et al, 2004), we proposed that treating tumors with high levels of GM-CSF would alter the activity of macrophages within the tumor microenvironment, stimulating them to secrete soluble VEGF receptor-1, whose endogenous role is to block angiogenesis. Since tumors require the exchange of nutrients and waste to grow beyond $2mm^3$, we suspected that the overall effects of this blockade could be monitored by reduced tumor growth. To test our hypothesis, we transplanted breast cancer cells into the breasts of normal FVB/N mice. When tumors became palpable, we randomized the mice to treatment groups. Mice received intratumor injections of 0, 1, 10, 100, or 1000ng of GM-CSF three times per week for three weeks. Once a week, tumor measurements were recorded.

Mice treated with increasing doses of GM-CSF demonstrated increasingly slowed rates of tumor growth, up to 100ng (**Figure 2A**). In these mice, there were no significant changes in body weight or overt clinical symptoms. CBCs with differential analysis performed at study end showed no significant changes in blood compartments, even at the 100ng dose (data not shown). Mice treated with 1000ng showed clinical signs such as ruffled fur and lethargy with two out of three mice dying spontaneously before study end. Based on these results, all subsequent studies used a 100ng dose.

To validate this method, 10 and 12 mice were treated with intratumor injections of PBS (vehicle) or 100ng GM-CSF three times per week. The results of tumor growth measurements are shown in **Figure 2B**. GM-CSF treatment significantly slowed tumor growth, demonstrating a 50% reduction in tumor diameter (representing a 75% reduction in tumor volume) at four weeks. These results were significant at a level of $p < 0.001$.

*GM-CSF treatment correlates with reduced lung metastasis***.** Malignancy is determined by the ability of cancer cells to metastasize to sights such as the lung, brain, thyroid, and bone. The PyMT model is an aggressive breast cancer that metastasize readily to the lung (Lin, E. Y. et al., 2003). Because tumor angiogenesis is an essential component of metastases and GM-CSF treatment induces an environment contrary to new blood vessel formation, we speculated that any reduction in angiogenesis would correlate with a reduction in metastases of these tumors to the lung. We orthotopically injected PyMT tumor cells into a single mammary gland of normal FVB/N female mice. Upon palpation, the mice were randomized into treatment groups and injected with PBS or 100 ng rmGM-CSF three times a week for eight weeks. After eight weeks, the mice were sacrificed, the lungs insufflated, removed, fixed and stained with eosin to identify solid masses within the lungs (**Figure 3A**). Quantification of tumor incidence indicates that GM-CSF treatment exudes a marked reduction in lung metastases of the mammary tumors compared to PBS treatment (P=0.061).

*Treatment lowers oxygen levels within the tumor proper***.** Because we observe reduced tumor growth rates in response to GM-CSF compared to PBS, and because GM-CSF induces the production of a potent inhibitor to angiogenesis, sVEGFR-1, from tumor-associated macrophages (Eubank, et al, 2004), we hypothesized that GM-CSF treatment may reduce the availability of oxygen within the tumor by disrupting new blood vessel formation. To track

changes in oxygen concentration in real-time within the tumors of GM-CSF-treated mice, we mixed lithium octa-n-butoxy 2,3-naphthalocyanine (LiNc-BuO) microcrystals with PyMT tumor cells and injected this mixture into the mammary fat pads of normal FVB/N female mice. 2-dimensional electron paramagnetic resonance (EPR) analysis was performed on the day of initial injection and once per week, subsequently, for four weeks. A higher resolution, 3-dimensional EPR reading was taken after three weeks. Mice were sacrificed to obtain anoxic control values after the fourth treatment week. Our data suggest that treatment with GM-CSF inhibits oxygenation of the tumor with the largest effect seen after 2 weeks of treatment (**Figure 4A**). 3-D EPR imaging of PBS- and GM-CSF-treated tumors after 3 weeks illustrate a reduction in the concentration of oxygen throughout the GM-CSF-treated tumors, but especially near the centers (**Figure 4B**). Distribution mapping showed the probe distribution to be uniform throughout the tumor, equal between groups, and limited to the tumor itself (data not shown).

GM-CSF causes increased necrosis within the tumor and changes in necrotic patterns. Depriving tumors of oxygen and other nutrients should cause cell death and tissue necrosis. Histologic evaluation of tumor sections validated the biologic significance of lower oxygen levels, as tumors treated with GM-CSF had higher levels of tissue necrosis (**Figure 5**). Tumor sections stained with H&E were viewed under a microscope using a 1X objective and laser/filter settings appropriate for green fluorescence. Under such conditions, necrotic tissue within H&E sections will autofluoresce. This allows for quantification of tumor necrosis as outlined in METHODS. Of particular note are the patterns of necrosis observed in the two different tissues. Mice treated with PBS demonstrate typical focal patterns of necrosis with a single area of involvement near the outer surface of the tumor (**Figure 5A**). In contrast, GM-CSF-treated

tumors reveal diffuse, multifocal patterns of necrosis, with smaller necrotic foci distributed throughout the tumor.

*sVEGFR expression mediates the effects of GM-CSF treatment***.** We hypothesized that GM-CSF treatment would induce tumor-associated macrophages to express sVEGFR-1, limiting angiogenesis and inhibiting tumor growth. To test this hypothesis, we orthotopically injected PyMT tumor cells into the mammary fat pad of normal FVB/N mice. Upon formation of a palpable tumor, the mice were randomized into the following four treatment groups: PBS, GM-CSF, GM-CSF plus a neutralizing antibody to sVEGFR-1, or GM-CSF plus an isotype IgG control antibody.

Treatment with a neutralizing antibody to sVEGFR-1 caused GM-CSF treated tumors to grow at rates nearly identical to vehicle-treated tumors (**Figure 6,** $p = 0.829$ vs PBS). Growth lines for GM-CSF treatment alone and GM-CSF plus isotype antibody were virtually superimposable, but much slower than the other two groups ($p = 0.025$ and 0.046 vs PBS, respectively).

DISCUSSION

We have proposed a model whereby one might change the behavior of cells within a tumor by therapeutically altering the factors present in the microenvironment. Such an approach to cancer therapy requires that we understand mechanisms of "tumor education" and that we identify factors which can stimulate cells within the tumor to adopt behaviors that will counter undesirable biological effects of that "education." This paper demonstrates our ability to do just that. Here we show that we can change the activity of macrophages to affect desirable outcomes in a murine model of breast cancer by local administration of a factor which causes a shift toward a more anti-tumor phenotype.

These data demonstrate how GM-CSF treatment slows tumor growth and prevents lung metastasis in our mouse model. In these studies we have used *in vivo*, intratumor oxygen measurement by EPR as a surrogate for angiogenic activity. In a normal, "healthy" tumor, growth results in escalating oxygen demand. This demand must be met by production of angiogenic factors coupled with new blood vessel growth. EPR analysis has allowed us to track changes in oxygenation throughout treatment. These studies (**Figure 4**) show that GM-CSF reduces oxygen levels within the tumors by depleting them of a major angiogenic factor, VEGF. Altered patterns of necrosis found in GM-CSF-treated tumors (**Figure 5**) illustrate the biological significance of the oxygen deprivation, and suggest that this blockade of angiogenic activity mediates the gross effects associated with intervention. The use of a neutralizing antibody to the soluble receptor (**Figure 6**) proves that this important anti-angiogenic factor largely mediates the effects of GM-CSF treatment. Since the IgG control treated group was not significantly different than the GM-CSF-treated group, it appears that this effect is related to the specific activity of the anti-sVEGFR-1 antibody and not to an antibody-mediated immune response. Taken together, these data show that GM-CSF slows tumor growth by starving them of oxygen and nutrients by increasing levels of sVEGFR-1 within the tumor.

We suspect that this anti-angiogenic activity can be attributed to a change in the phenotype of macrophages within the tumor, similar to the changes we observed *in vitro*, marked by the expression of sVEGFR-1. Studies aimed at characterizing the gene expression patterns of tumor-associated macrophages, neutrophils, and other cells will shed more light on the cell types that produce the sVEGFR-1 upon treatment with GM-CSF.

Tumor metastasis also depends on blood vessel growth. Not only do vessels provide a route for escape by hematogenous spread, but the processes required for invasion of vessels into the tumor

can facilitate detachment of cells from the surrounding tissues and entrance into the blood stream (WEIDNER, N. et al., 1991). We presume at this time that reductions in lung metastases we observe (**Figure 3**) are a direct result of impaired angiogenic activity. However, we have also found significant changes in other factors within the tumor which might affect rates of tumor metastasis (data not shown). Further studies will be needed to show the relative contribution of each to metastasis.

We have shown that it is possible to reprogram the tumor microenvironment and change the way "tumor educated" cells behave *in vivo* using therapeutically-plausible techniques. Such models provide a powerful platform for studying the involvement of macrophages and other cells in the processes which comprise malignant transformation. Detailed studies of the changes invoked by treatment within each cell type and within the tumor organ will help us understand the role each cell type plays in malignant conversion and possibly identify novel therapeutic targets.

REFERENCES

Achilles, E. G., Fernandez, A., Allred, E. N., Kisker, O., Udagawa, T., Beecken, W. D., Flynn, E., and Folkman, J. 2001. Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for"no take"of human tumors in mice. Journal of the National Cancer Institute 93.1075.

Armstrong, C. A., Botella, R., Galloway, T. H., Murray, N., Kramp, J. M., and Song, I. S. 1996. Antitumor effects of granulocyte-macrophage colony-stimulating factor production by melanoma cells. Cancer Research 56.2191-8.

Demirkesen, C., Buyukpinarbasili, N., Ramazanoglu, R., Oguz, O., Mandel, N. M., and Kaner, G. 2006. The correlation of angiogenesis with metastasis in primary cutaneous melanoma: a comparative analysis of microvessel density, expression of vascular endothelial growth factor and basic fibroblastic growth factor. Pathology 38.132.

Eubank, T. D., Galloway, M., Montague, C. M., Waldman, W. J., and Marsh, C. B. 2003. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. J Immunol 171.2637-43.

Eubank, T. D., Roberts, R., Galloway, M., Wang, Y., Cohn, D. E., and Marsh, C. B. 2004. GM-CSF induces expression of soluble VEGF receptor-1 from human monocytes and inhibits angiogenesis in mice. Immunity. 21.831-42.

Folkman, J. 1990. What is the evidence that tumors are angiogenesis dependent? Journal of the National Cancer Institute 82.4.

Hagemann, T., Robinson, S. C., Schulz, M., Trumper, L., Balkwill, F. R., and Binder, C. 2004. Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. Carcinogenesis 25.1543-9.

Hanahan, D. and Weinberg, R. A. 2000. The hallmarks of cancer. Cell (Cambridge) 100.57.

Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., and Pollard, J. W. 2003. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases1. Am J Pathol 163.2113-26.

Lin, E. Y., Nguyen, A. V., Russell, R. G., and Pollard, J. W. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193.727-40.

Lotem, J. and Sachs, L. 2002. Cytokine control of developmental programs in normal hematopoiesis and leukemia. Oncogene 21.3284-94.

Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M., and Mori, T. 1993. Expression of E-Cadherin Cell-

Adhesion Molecules in Human Breast-Cancer Tissues and Its Relationship to Metastasis. Cancer Research 53.1696-701.

Pollard, J. W. 1997. Role of colony-stimulating factor-1 in reproduction and development. Molecular Reproduction and Development 46.54.

Pollard, J. W. 2004. Tumour-educated macrophages promote tumour progression and metastasis. Nature reviews.Cancer 4.71.

Sapi, E. 2004. The role of CSF-1 in normal physiology of mammary gland and breast cancer: an update. Exp Biol Med (Maywood.) 229.1-11.

Tang, R., Beuvon, F., Ojeda, M., Mosseri, V., Pouillart, P., and Scholl, S. 1992. M-CSF (monocyte colony stimulating factor) and M-CSF receptor expression by breast tumour cells: M-CSF mediated recruitment of tumour infiltrating monocytes? J Cell Biochem 50.350-6.

Tsuruma, T., Yagihashi, A., Hirata, K., Torigoe, T., Araya, J., Watanabe, N., and Sato, N. 1999. Interleukin-10 reduces natural killer (NK) sensitivity of tumor cells by downregulating NK target structure expression. Cellular Immunology 198.103.

Watson, N. F., Ramage, J. M., Madjd, Z., Spendlove, I., Ellis, I. O., Scholefield, J. H., and Durrant, L. G. 2006. Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. International journal of cancer.Journal international du cancer 118.6.

WEIDNER, N., SEMPLE, J. P., WELCH, W. R., and Folkman, J. 1991. TUMOR ANGIOGENESIS AND METASTASIS - CORRELATION IN INVASIVE BREAST-CARCINOMA. NEW ENGLAND JOURNAL OF MEDICINE 324.1-8.

FIGURE LEGENDS

Figure 1. GM-CSF alters the expression of angiogenic molecules in monocytes.
Many breast tumors express M-CSF, which stimulates monocyte/macrophages to secrete VEGF, promoting a pro-angiogenic phenotype. GM-CSF, on the other hand, makes these cells produce sVEGFR, which can block vascular growth. sVEGFR will not only block the effects of VEGF produced by the monocytes themselves, but can bind VEGF from any source, including that produced by the tumor cells. This and other effects of GM-CSF may slow tumor growth and hinder metastasis. The underlying hypothesis of this paper is that local GM-CSF injection to create higher levels of GM-CSF within the tumor will change the phenotype of the macrophages within the tumor, causing them to adopt more anti-tumor, anti-angiogenic behaviors.

Figure 2. Intratumor GM-CSF injection slows tumor growth and prolongs survival.
(A) 8-10 week old female FVB/N mice were injected with 5 X 10^5 PyMT tumor cells into the #4 mammary fat pad. Upon palpable tumor formation, mice were randomized to one of five treatment groups, 0, 1, 10, 100, or 1000 ng. Mice were treated with this dose of GM-CSF by injection into the tumor 3X per week for three weeks. Tumor size (tumor diameter in the greatest dimension) and mouse weight was measured weekly. Mouse weight did not differ between groups for the duration of the study (data not shown). Data shown represent average tumor size from three mice per group.

(B) FVB/N mice were injected with PyMT tumor cells, as above. Upon tumor formation, mice were randomized to treatment (100 ng GM-CSF in PBS) or vehicle (PBS alone) groups. Mice were treated 3X per week until tumors reached 2 cm in their greatest diameter. Tumor size (diameter in the greatest dimension) and mouse weight was measured weekly. Mouse weight did not differ between groups (data not shown). Data shown represent 10 and 12 mice for vehicle

and treatment groups, respectively. Difference between groups is significant at the $p < 0.001$ level when compared by ANOVA.

Figure 3. Local GM-CSF treatment reduces tumor metastases to lung.
On euthanasia, lungs were removed from GM-CSF and PBS-treated mice, insufflated, fixed in formalin, and whole-mounted. These lungs were then imaged on a stereo microscope and tumor metastases noted.

(A) Representative images of lungs from GM-CSF and PBS-treated mice. Arrows show opacities in the lung which suggest metastatic tumor growth.

(B) Quantification of tumor metastases. Data represents mean number of metastases per mouse ±SEM over 7 mice per group.

Figure 4. Intratumor GM-CSF treatment reduces oxygen levels within mammary tumors in vivo.
(A) A subset of mice from studies shown in Figure 3 were given orthotopic injections of tumor cells containing nanoparticles of LiNC-BuO, allowing for real-time, *in vivo* oxygen measurement by electron paramagnetic resonanance imaging (EPR). Oxygen levels in these mice were measured weekly by 2D EPR, and once at 3 weeks by 3D EPR. Lines represent average oxygen concentration in mmHg taken from 2D EPR measurements ±SEM from 4 mice per group.

(B) Representative samples of 3D EPR imaging (2 mice from each group) showing oxygen distribution throughout the tumor. Measurements of nanoparticle density and distribution in the area around the tumor were made concurrently. These measurments showed that the nanoparticles were limited in space to the area corresponding to the tumor proper, with uniform distribution of the particles throughout the tumor (data not shown). These images were taken on mice treated with GM-CSF or PBS for 3 weeks.

Figure 5. GM-CSF treatment may change the pattern and amount of necrosis within tumors.
On euthanasia, tumors were removed from GM-CSF and PBS-treated mice, fixed in formalin, paraffin-embedded, sectioned, and stained with H&E. Sections were then imaged using a 1X objective under green fluorescence conditions. Under these conditions, necrotic tissue will autofluoresce, allowing for identification of necrotic and viable .

(A) Representative images of H&E-stained tumors under green fluorescence.

(B) Quantification of necrosis. Data represents average percentage of necrotic tissue (area of green autofluoresence over total tumor area). Numbers show mean ±SEM of 4 mice per group.

Figure 6. sVEGFR neutralization restores normal tumor growth patterns.
Normal female FVB/N mice were given orthotopic injections of 5 X 10^5 PyMT tumor cells as in Figure 3. Upon tumor formation, mice were randomized to receive injections of PBS vehicle or GM-CSF (as before), or GM-CSF plus a sVEGFR blocking antibody or isotype antibody. Intratumor injections were given 3 times weekly for 3 weeks. Graph shows growth of tumors as measured in their greatest diameter. Circles represent mean tumor size ± SEM for each group at each timepoint. Groups consisted of 4, 5, 8, and 4 mice, respectively. p values vs PBS group: GM alone = 0.025, GM + aSR = 0.829, GM + Isotype = 0.046 by repeated measures ANOVA with Tukey post hoc testing.

Figure 1.

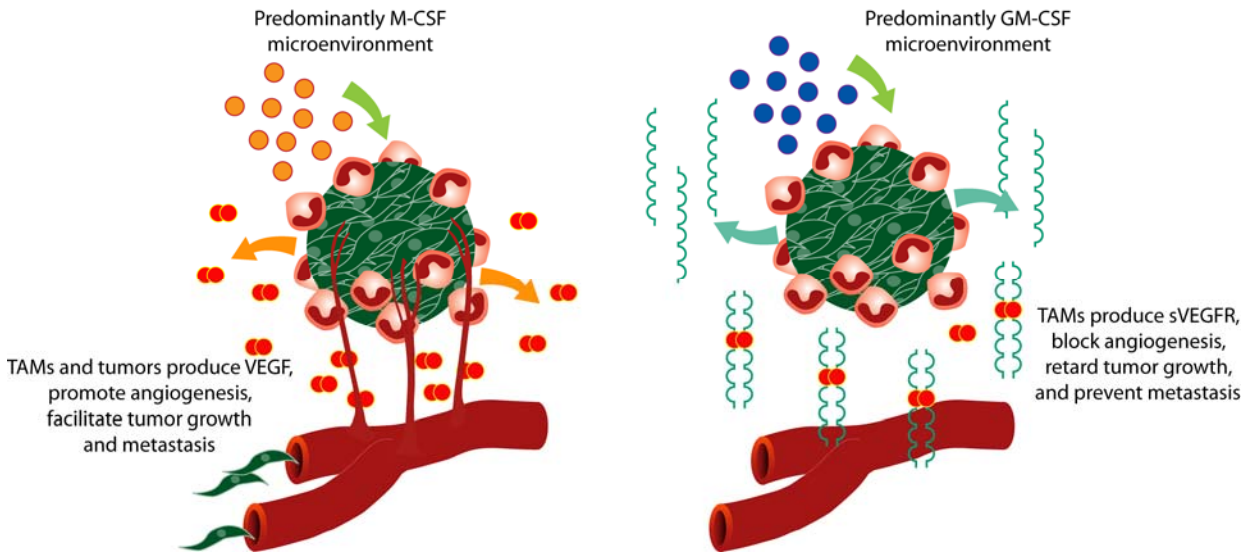


Figure 2.

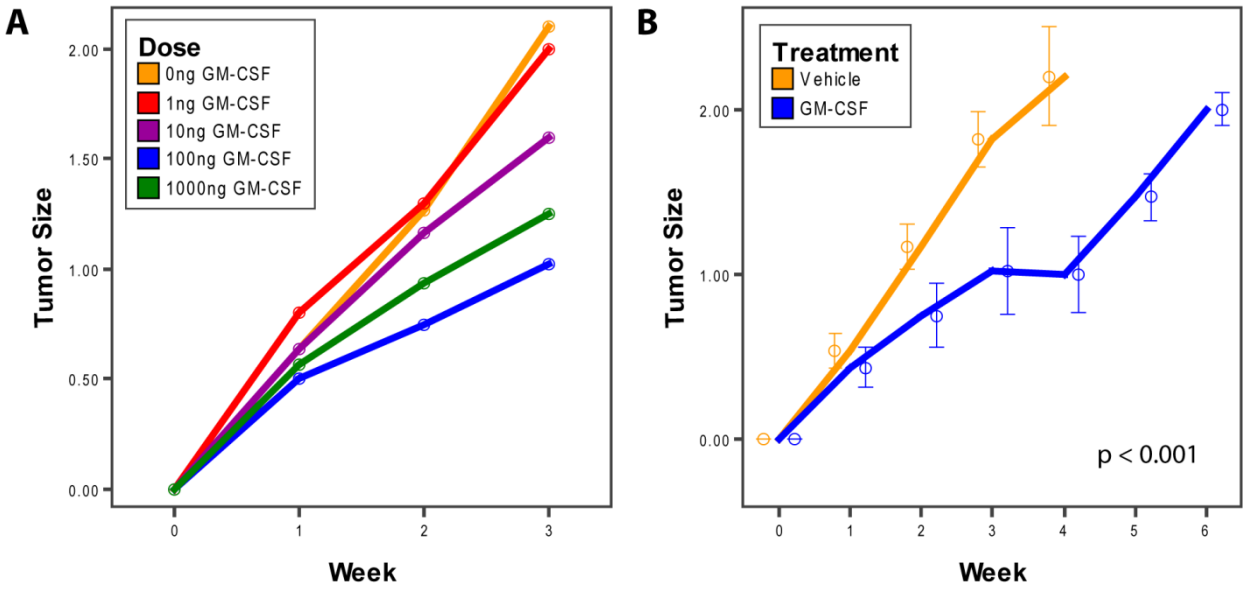
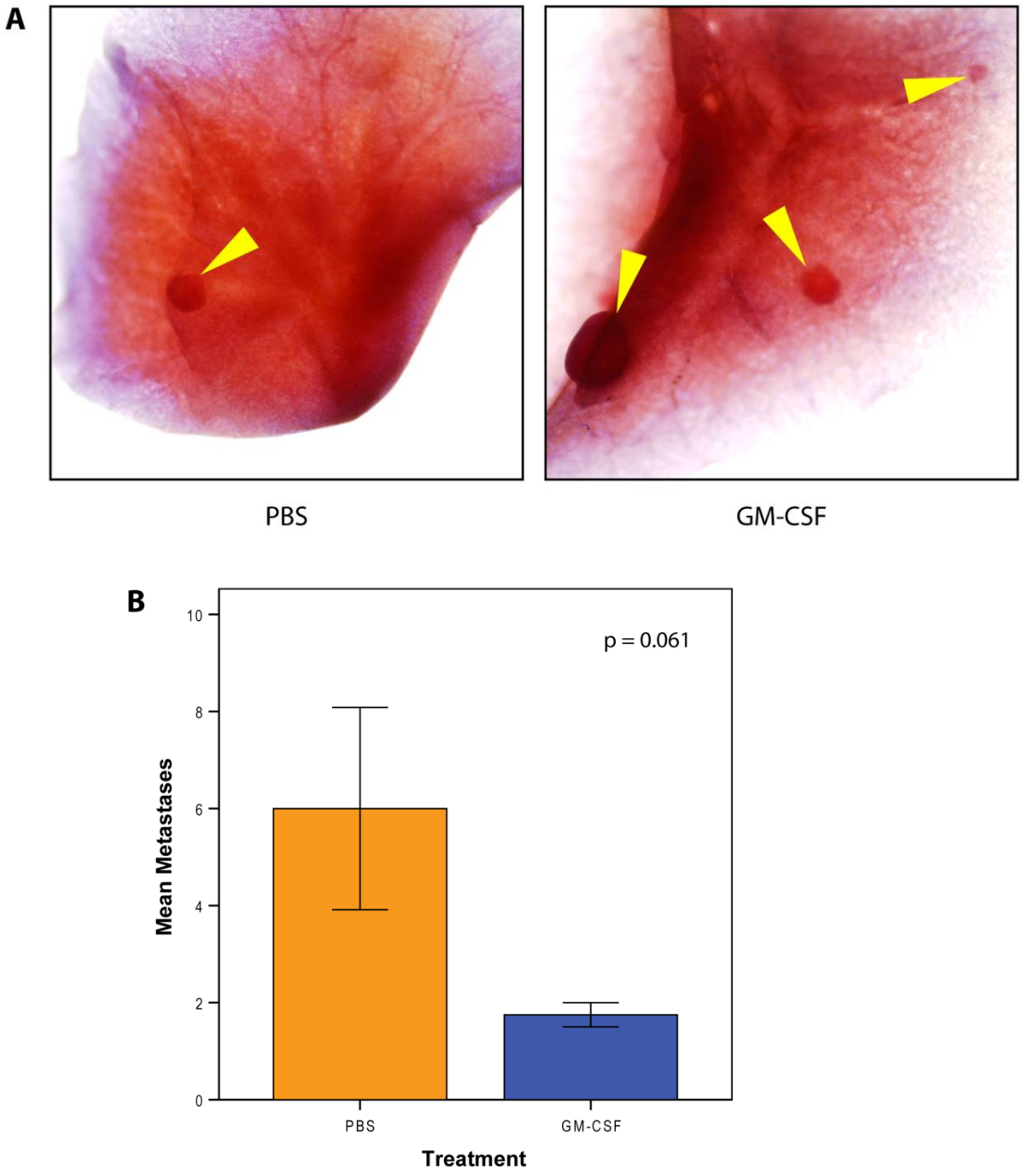


Figure 3.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PBS
GM-CSF
Treatment

Figure 4.

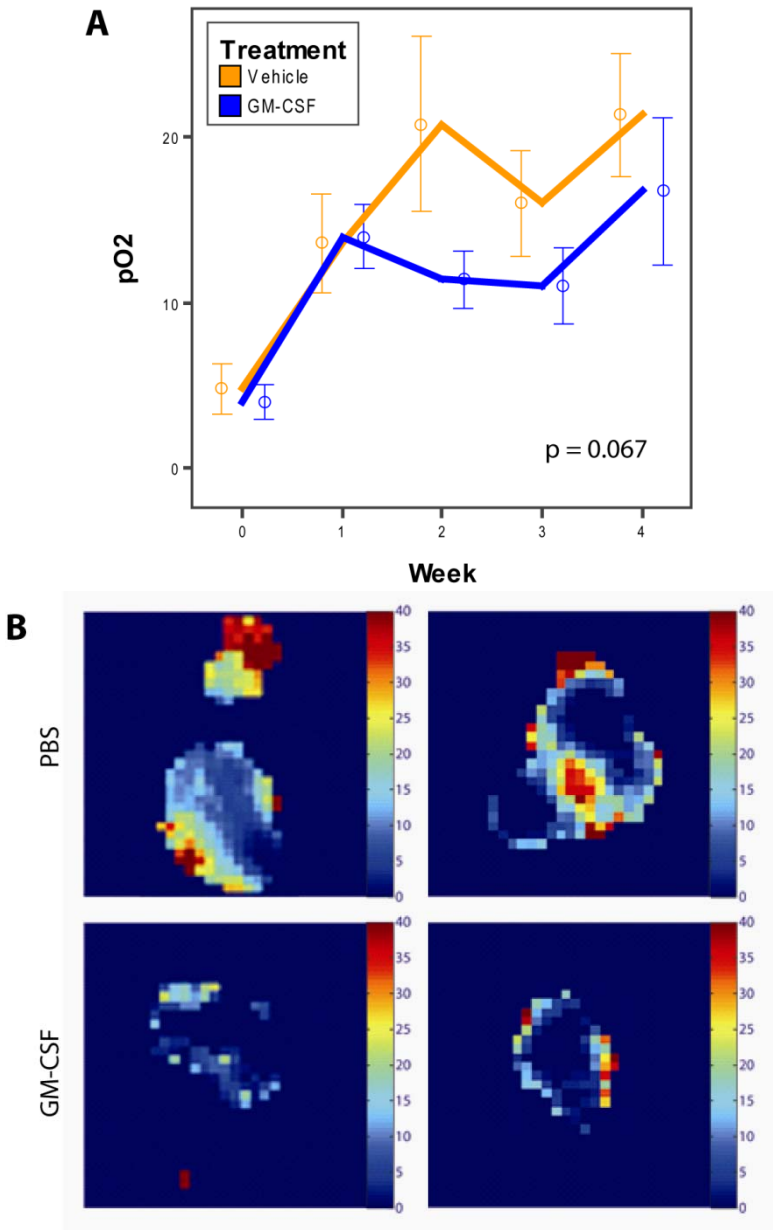
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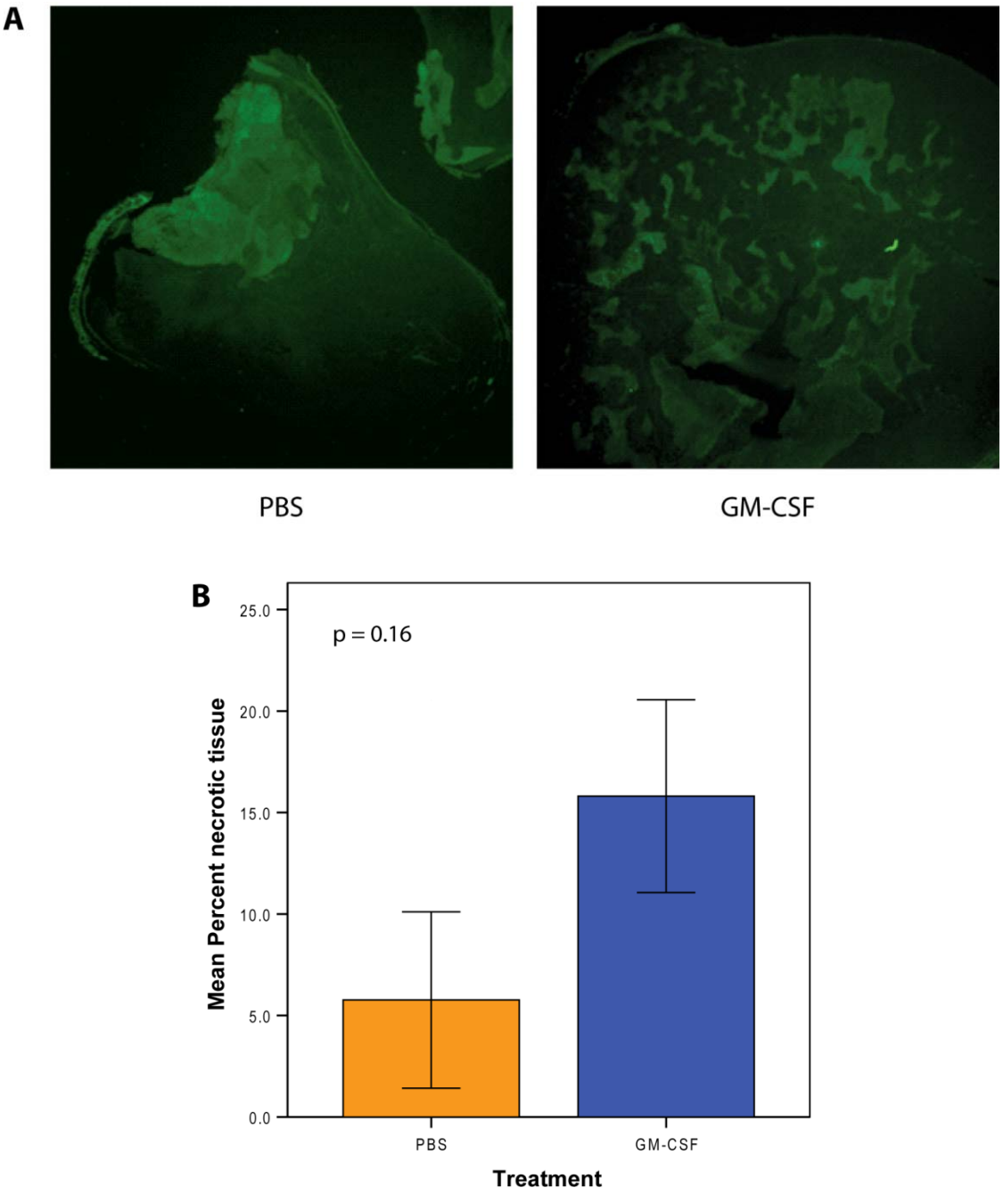
Figure 5.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B
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5.0
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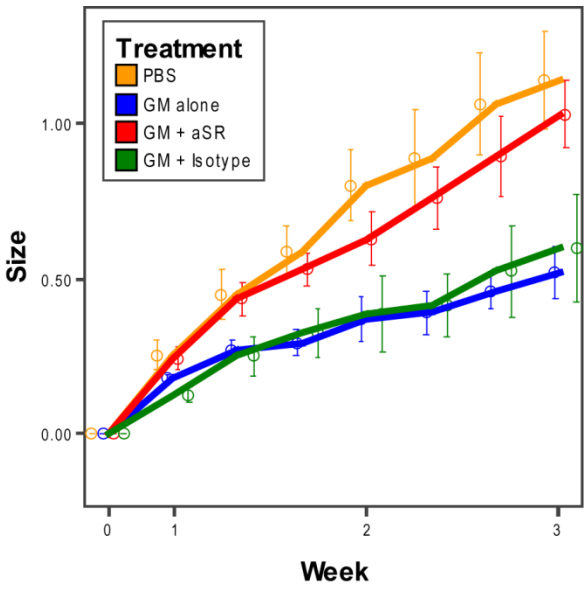
PBS
GM-CSF
Treatment

Figure 6.


Treatment
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GM alone
GM + aSR
GM + Isotype
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1
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